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Expression and Characterisation of the Heavy Chain of Tetanus Toxin: Reconstitution of the Fully-Recombinant Dichain Protein in Active Form[1]

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Tetanus toxin, composed of a disulphide-linked heavy (HC) and light (LC) chain, preferentially blocks the release of inhibitory neurotransmitters in the spinal cord by Zn^{2+}-dependent proteolytic cleavage of synaptobrevin. This intoxication involves binding *via* HC to ecto-acceptors on peripheral nerve endings, followed by internalisation and retrograde transportation to its prime site of action in central neurons. To facilitate exploitation of the toxin's unique activities, HC was expressed at a high level in *Escherichia coli* as a fusion with maltose binding protein; after cleavage by thrombin, free HC was isolated and its identity confirmed by Western blotting and N-terminal microsequencing. The expressed and native HC gave very similar circular dichroism spectra, excluding any gross differences in their folded structures. Recombinant HC antagonised the neuromuscular paralysing activity of the native toxin, by competing for binding to neuronal ecto-acceptors. The HC was reconstituted with bacterially-expressed LC to create disulphide-bridged dichain toxin that blocked neuromuscular transmission. The fully-recombinant toxin produced spastic paralysis in mice characteristic of the blockade of central inhibitory synapses, revealing that it undergoes axonal transport to the spinal cord, like the native toxin but with a reduced efficacy. This first report of the large-scale production of recombinant tetanus toxin in active form should facilitate studies on the use of engineered innocuous forms of the toxin as neuronal transport vehicles.

Key words: ecto-acceptors, expression, heavy chain, reconstitution, tetanus toxin.

Tetanus toxin (TeTx), produced by the strictly anaerobic, spore-forming rod-shaped bacillus *Clostridium tetani*, causes spastic paralysis by blocking neurotransmitter release at inhibitory synapses (*1*). This protein is synthesised as a single chain (~150 kDa) which is later cleaved by bacterial proteases to produce a dichain species, composed of a heavy (HC; ~100 kDa) and a light (LC; ~50 kDa) chain that are held together by strong non-covalent interactions and an inter-chain disulphide bridge (*2*). Although TeTx preferentially blocks the release of inhibitory transmitters in the central nervous system (CNS), at high concentration it acts at the neuromuscular junction (NMJ) preventing the release of acetylcholine and causing flaccid paralysis (*3-6*). The action of TeTx involves a sequence of several steps (*7*): specific binding to neuronal ecto-acceptors, internalisation, translocation to the cytosol and, finally, inhibition of exocytosis due to cleavage of synaptobrevin by the Zn^{2+}-dependent protease activity of its LC (*8, 9*) and, to some extent, by a protease-independent activation of neuronal transglutaminase(s) (*10, 11*).

The two constituent chains of TeTx can be separated with retention of their biological activities; also, when reconstituted they form the disulphide linked dichain toxin (*2*). Treatment of the isolated HC with papain (*12*) generates H_C (~50 kD; C-terminal half of HC) and H_N (~50 kD; N-terminal half of HC); these two fragments and LC appear to represent functional domains that are concerned with the multi-phasic intoxication process. HC or H_C can compete with TeTx for binding to its acceptors on cultured cells (*13-15*) and antagonise the neuromuscular paralytic action of TeTx (*13, 14*). Also, H_C is known to bind to certain gangliosides on the surface of target cells (*15, 16*) and undergo retrograde axonal transport, albeit to a limited extent (*17*), whilst H_N has been shown to form channels in cell membranes (*18*) and contribute to the translocation of TeTx across membranes (*19*). LC is responsible for the intracellular blockade of transmitter release (*8, 9, 20, 21*).

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Abbreviations: TeTx, tetanus toxin; LC and HC, light and heavy chains of TeTx; H_C and H_N, C- and N-terminal halves of HC; LD_{50}, amount of toxin that kills half of the injected mice within 4 days; factor Xa, activated factor X protease; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MBP, maltose binding protein; MCS, multiple cloning site; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; LB, Luria-Bertani medium; LA, Luria-Bertani agar; CNS, central nervous system; NMJ, neuromuscular junction; BSA, bovine serum albumen; KR and mKR, normal and modified Krebs Ringer solution.

After the initial cloning of TeTx gene (*22*), H_C was successfully expressed in *Escherichia coli* (*23*), yeast cells (*24*), and insect cultured cells (*25*). The recombinant H_C (r-H_C) was shown to bind to ganglioside (*26*) and to protect mice against a challenge with TeTx (*27, 28*). There have been numerous reports on the ability of H_C to transport exogenous proteins into CNS (*29-32*). These demonstrated that large proteins, whose entry to CNS is limited by the blood-brain barrier, may gain access through endocytosis in the periphery when conjugated to H_C. Moreover, attachment of H_C to horseradish peroxidase seemed to enhance the stability of this protein (*31*), as well as promoting its movement by trans-synaptic transport. However, whole TeTx binds with far higher affinity to neurons than H_C and is also much more efficient in undergoing retrograde axonal transport, under physiological conditions (*2, 17*), indicating that H_N fragment and/or LC of the toxin may contribute indirectly to the binding and transport of TeTx.

Although active recombinant LC (r-LC) has been successfully produced in *E. coli* (*33, 34*), the preparation of r-HC has not been reported, even though its use instead of native HC is particularly important for reconstitution with enzymically-inactive mutated LC (*33*) to form an innocuous dichain which has great potential as a novel transporter. Therefore, a fusion of HC with maltose binding protein (MBP) was expressed in *E. coli* and, after purification f free r-HC, its properties were compared with those of H_C prepared in similar manner. As fully-recombinant TeTx would be useful for elucidating the mechanisms underlying its acceptor-mediated internalisation and retrograde transport, this was obtained, for the first time, by reconstitution of expressed LC with r-HC and the resultant toxin shown to block neuroexocytosis and induce spastic paralysis.

MATERIALS AND METHODS

Materials—Restriction endonucleases, DNA modifying enzymes and DNA purification kits were from Promega and used as recommended by the manufacturer. pMAL-c2 vector containing a factor Xa site was purchased from New England Bio-Labs; pMAL-c2-T is a modified version of the latter produced by insertion of a histidine tag and a thrombin susceptible site (see later). Thrombin, factor Xa and Sepharose 6B were obtained from Sigma. Purified

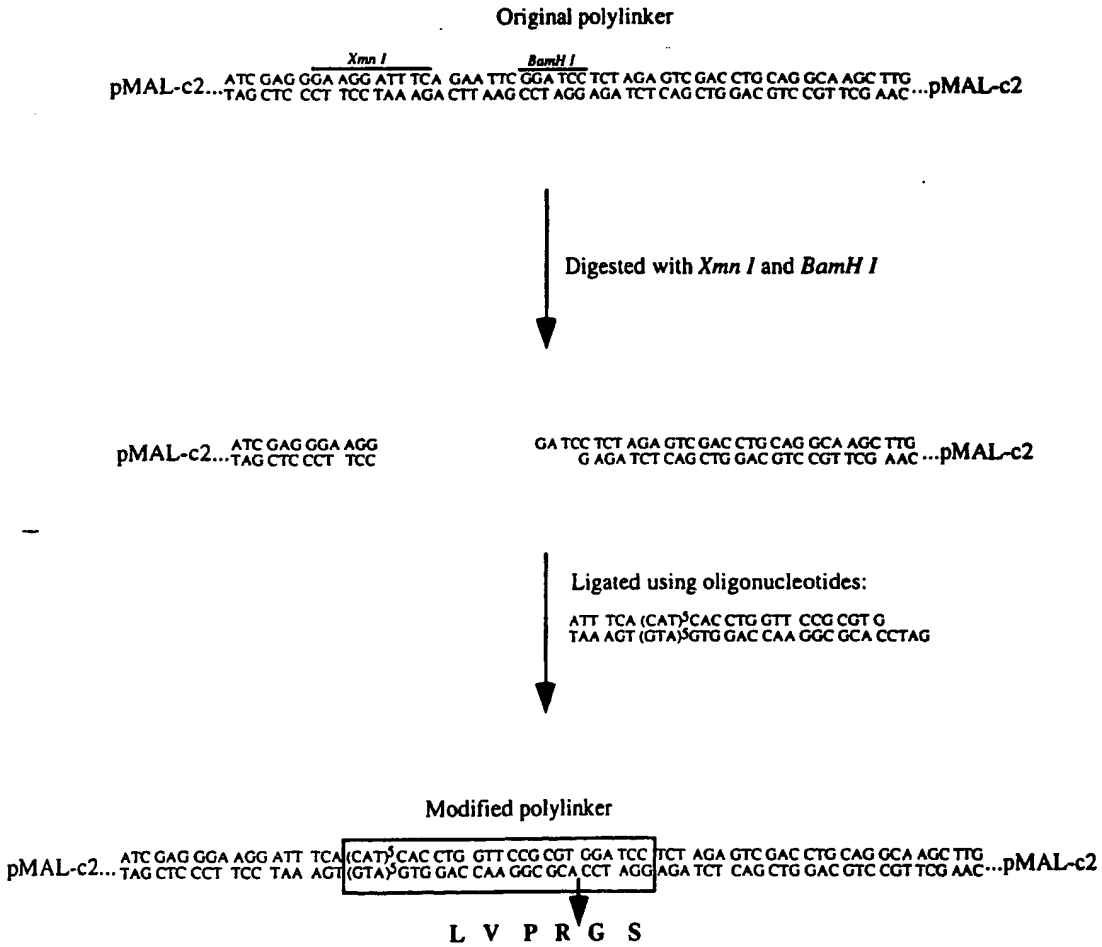

Fig. 1. **Schematic representation of the construction of the expression vector pMAL-c2-T by insertion of a modified polylinker into pMAL-c2.** The original polylinker was removed from pMAL-c2 by digestion with restriction-enzymes *Xmn*I and *Bam*HI. The resultant fragments were ligated using the two specified oligonucleotides to generate a modified polylinker (boxed), containing six consecutive histidines [(CAT)^5 CAC] and a thrombin site. The product was subcloned into pMAL-c2, cut with the above-noted enzymes, and the sequence of the new vector pMAL-c2-T verified by DNA sequencing. The amino acid sequence recognised by thrombin is shown together with the peptide bond cleaved (↓).

TeTx, native LC, and HC were kindly provided by Dr. U. Weller.

Preparation of pMAL-c2-T—The vector pMAL-c2 has been widely used to express in *E. coli* foreign proteins fused with MBP *via* a factor Xa cleavage site in this laboratory (*33, 35*). As factor Xa is expensive and relatively inefficient in cleaving MBP from its fusion with HC of TeTx (unpublished observation), this vector was improved by introducing nucleotide sequences encoding a thrombin site and six consecutive histidines into the multiple cloning site (MCS) of pMAL-c2. These modifications were made as outlined in Fig. 1. Briefly, two oligonucleotides corresponding to the sense and antisense sequence of the polylinker were synthesised, phosphorylated and mixed together at 100°C for 5 min; annealing was promoted by cooling down the mixture slowly (less than 1°C/min) to 22°C. About 0.1 μg of the annealed polylinker was ligated with 10-20 ng of pMAL-c2 cut by *Xmn*I and *Bam*HI at 15°C for 16 h. The product was transformed into *E. coli* TG1 strain and spread onto Luria-Bertani agar (LA) plates containing 100 μg/ml of ampicillin; colonies containing the new polylinker in pMAL-c2 were confirmed by DNA sequencing of the modified junction in the isolated vector, using Sequenase version 2.0 DNA sequencing kit (USB).

Construction of MBP-HC and MBP-H_C Plasmids—Oligonucleotides used as primers in the amplification of HC and H_C genes were synthesised on a DNA/RNA synthesiser (Applied Biosystems). The whole HC gene was created by ligating its three fragments from pTet8, pTet14 and pTet215; these had been cloned into different vectors and were kindly provided by Dr. N.F. Fairweather. First, the gene encoding H_N was amplified by PCR using primers *a* (5′-AAT AGA TCT AGA TCA TTA ACA GAT TTA GGA-3′) and *b* (5′-TTC TAA AGA TCT ATA CAT TTG ATA ACT-3′) from pTet8; primers *c* (5′-ATG TAT AGA TCT TTA GAA TAT CAA GTA-3′) and *d* (5′-ATC GAT AAG CTT *TTA TCA* GTC GAC CCA ACA ATC CAG ATT TTT AGA-3′) from pTet14. The two PCR products were joined together by ligation *via* the *Bgl*II (AGA TCT) site, creating the H_N gene product. H_N DNA was cut by *Xba*I (TCT AGA) and *Hin*dIII (AAG CTT) and subcloned into pMAL-c2-T which had been cut with the same two enzymes, generating pMAL-H_N plasmid. The whole HC gen was produced by joining the following fragments together: (i) H_N released from pMAL-H_N plasmid by *Sac*I (GAG CTC) and *Sal*I (GTC GAC); (ii) H_C cut by *Sal*I and *Bam*HI from plasmid pTet215; and (iii) *Sac*I and *Bam*HI cut pMALc2-T vector, creating the pMAL-HC plasmid. For the preparation of pMAL-H_C construct, primers *e* (5′-AAT AGA GGA TCC AAC GAA GAA GAC ATC-3′) and *f* (5′-GCT AGC TCT AGA *TTA TCA* GTC GTT GGT-3′) were employed to amplify its coding sequence from pTet215. The *Bam*HI (GGA TCC) and *Xba*I (TCT AGA) digested PCR product was subcloned into a pMAL-c2 cut with same pair of enzymes. The ligation mixture was incubated at 15°C for 16 h. Competent *E. coli* cells (JM109 strain for pMAL-HC and TG1 strain for pMAL-H_C) prepared in ice-cold 75 mM $CaCl_2$ buffer were used for transformation. Recombinant clones were screened on LA plates containing 100 μg/ml of ampicillin and the identity of the junction in the DNA insert was confirmed by sequencing. The pMAL-HC possesses a sequence encoding six consecu-

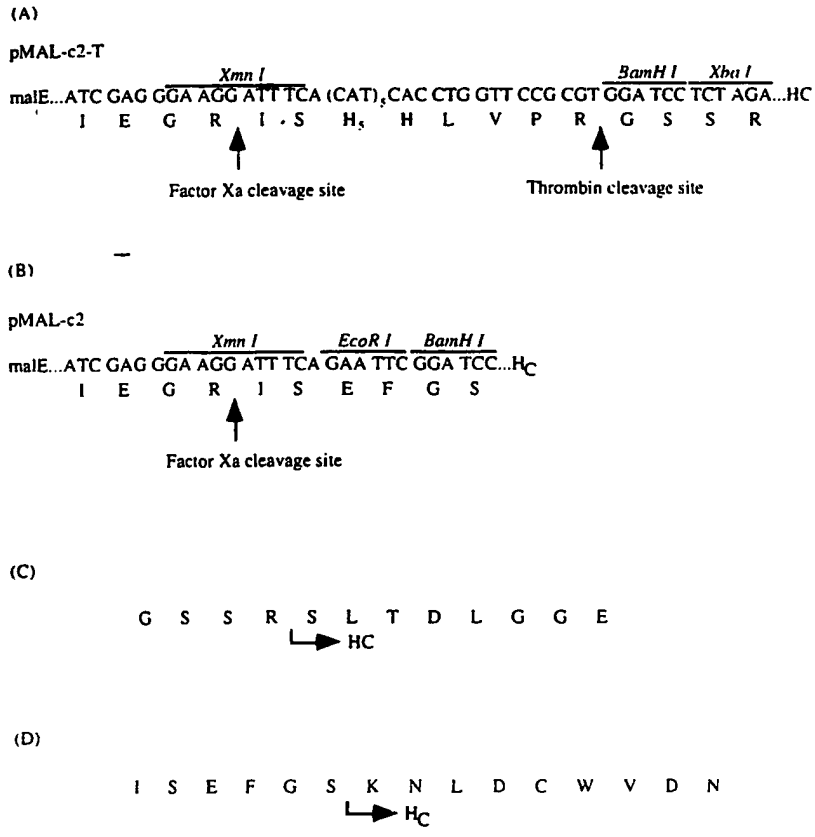

Fig. 2. **Schematic presentation of pMAL-HC and pMAL-H_C used for expressing r-HC and r-H_C: verification of the N-terminal sequence of r-HC and r-H_C.** (A) Illustrates the MCS of pMAL-c2-T, including the factor Xa cleavage site, six consecutive histidines (His-tag) and thrombin cleavage site, which links MBP and HC. (B) Represents MCS of pMAL-c2, including the factor Xa cleavage site, between MBP and H_C. (C) Shows the first 12 residues found at the N-terminus of r-HC protein purified from *E. coli*; microsequencing proved that the eight residues shown (arrow) at the N-terminus of r-HC are identical to those of native HC, preceded by the four amino acids encoded by the MCS sequence of pMAL-c2-T. (D) Represents the first 15 amino acids of the sequence obtained for the N-terminus of r-H_C: nine of these amino acids (arrow) are the same as those in native H_C, preceded by the six residues encoded by the MCS sequence of pMAL-c2.

tive histidines and cleavage sites for factor Xa and thrombin (Fig. 2A) whereas the pMAL-H_C construct contains a factor Xa cleavage site sequence between MBP and H_C protein (Fig. 2B).

Preparation of Amylose Affinity Resin by Coupling of Starch to Sepharose 6B—This was achieved using a modified protocol from Hermanson *et al.* (*36*). Sepharose 6B (100 ml) was washed with 2 liters of distilled water and then in 200 ml of 1 M Na_2CO_3 (pH 11) before being resuspended in 40 ml of the latter buffer. Five milliliters of divinylsulfone was added and the suspension slowly mixed at 22°C for 60 min. The activated matrix was washed with 4 liters of distilled water and then suspended in 100 ml of 6% (w/v) soluble starch in 1 M Na_2CO_3. The mixture was continuously rotated at 50°C for 1 h before the addition of 5 ml of 2-mercaptoethanol to quench the unreacted divinylsulfone. After 30 min at 22°C, the resin was washed with 1 liter of column buffer [10 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, and 1 mM dithiothreitol (DTT)]. The resin was stored in 0.05% (w/v) sodium azide at 4°C before use.

Expression and Purification of r-HC and r-H_C Proteins—Overnight cultures of bacteria containing either pMAL-HC or pMAL-H_C construct was diluted (1:100) into fresh Luria-Bertani medium (LB) containing ampicillin (100 μg/ml), and grown for 2-2.5 h with shaking (200 rpm) at 37°C to reach the appropriate cell density (A_{600}~0.5). Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM and the cultures shaken for an additional 3 h at 37°C (for r-H_C) and 16-20 h at 30°C (for r-HC). The cells were harvested by centrifugation at 6,000 × *g* for 30 min at 4°C and resultant pellets were resuspended in the column buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) before lysis by sonication for 10 min (10 s pulses plus an interval of 30 s) on setting 10 (maximal scale; Ultrasonic Processor, Model XL2020, Heat System, UK). After centrifugation of the cell lysate at 9,000 × *g* for 30 min at 4°C. the supernatants were loaded onto amylose affinity columns (2.5 × 10 cm, 40 ml resin) equilibrated with the column buffer. Following removal of unbound proteins by washing with the column buffer (320 ml), the bound MBP-HC or MBP-H_C fusion protein was eluted with the same buffer containing 10 mM maltose. The fusion proteins were concentrated and cleaved either by thrombin (for MBP-HC) at 4°C for 16 h (1 unit to 75 μg of the fusion protein) or by factor Xa (for MBP-H_C) at 22°C for 24 h [at an enzyme:protein ratio of 0.5-1:100 (w/w)]. The digestion was terminated by adding PMSF (final concentration 1 mM) to inactivate the excess proteases and the reaction mixture analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The cleaved fusion proteins, after dialysis against the column buffer to remove the free maltose, were isolated from free MBP by readsorption of the latter to a new amylose column. The purified r-HC or r-H_C were analysed by SDS-PAGE and Western blotting. N-terminal sequencing of r-HC and r-H_C was carried out by subjecting the sample to SDS-PAGE, transfer onto polyvinylidene difluoride membranes (*37*) and automated Edman degradation using a model 4000 protein sequencer (Chelsea Instruments, London).

CD Measurement of Native HC and r-HC—Far-UV CD spectra were recorded on an Aviv 62DS, with an acceptance angle ≥ 90°, fitted with a thermostated cell holder, using one 1 mm pathlength suprasil cuvette. Data points were collected in steps of 0.2 nm, from 300 to 190 nm. The CD spectra were recorded at 22°C at concentrations of native HC and r-HC at 0.10 mg/ml after dialysis against 10 mM sodium phosphate buffer (pH 7.0) at 4°C f r 24 h. Each CD spectrum is the average of 5 scans from which the c rresponding averaged baseline spectrum, obtained under identical conditions, has been subtracted. The results are expressed as mean residue ellipticity, taking a mean residue molecular mass for HC of 114.7, which was calculated from its amino acid sequence (*38*). Data were analysed to estimate the percentage of secondary structure, using a least squares algorithm (*39*) with reference data from Chang *et al.* (*40*).

Measurement of the Effect of Native HC, r-HC, or r-H_C on the Blockade by TeTx of Neuromuscular Transmission—Mouse left phrenic nerve-hemidiaphragm, dissected from mice (T/O strain, 4 weeks old and ~20 g in weight), was immediately transferred into a closed circulatory superfusion system containing 10 ml of a modified Krebs-Ringer solution [mKR, consisting of: 118 mM NaCl, 4.7 mM KCl, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 23.8 mM $NaHCO_4$, 1.2 mM KH_2PO_4, 11.7 mM glucose, pH 7.4 (*41, 42*)] bubbled with 95% O_2 and 5% CO_2, and supplemented with 0.1% (w/v) bovine serum albumen (BSA) to diminish n nspecific adsorption of the toxins. The tissues were first incubated in mKR buffer alone (as a control) or the latter containing 500 nM native HC, 1 μM r-HC, 2 μM r-H_C, or 2 μM MBP, at 4°C for 60 min; 1 nM TeTx was then add d into the bath and co-incubated at 4°C for a further 30 min. To determine whether these proteins could displace bound TeTx, 1 nM TeTx was first incubated with the diaphragm in mKR buffer at 4°C for 30 min, followed by incubati n without (as a control) or with 500 nM native HC, 1 μM r-HC, or 2 μM r-H_C for a further 60 min. The diaphragms, after being washed thrice with mKR and with KR buffer (mKR with 2.5 mM $CaCl_2$ and 1.2 mM $MgSO_4$) for another three times, were kept in a bath containing 10 ml KR buffer before the temperature was raised to 37°C. Muscle twitch was evoked by supra-maximal stimulation (Grass model S48 stimulator) of the phrenic nerve with bipolar electrodes and recorded *via* a force-displacement transducer (Lectromed, UK) connected to an amplifier and computer system (MacLab, AD Instruments, UK). Parameters of nerve stimulation were 0.2 Hz square waves of 0.1 milliseconds duration with 1.5-2.5 V amplitude. Toxin-induced paralysis of neuromuscular transmission was calculated as the time taken for nerve-evoked muscle contraction to decrease to 10% of original value, as described previously (*43*).

Reconstitution of Native and Recombinant Dichains and Assessment of Their Biological Activities—LC of TeTx was expressed in *E. coli* as a fusion with MBP and purified on amylose resin; after cleavage with protease, LC was separated from MBP by affinity chromatography as detailed previously (*33*). Reconstitution was achieved by mixing equimolar amount of native (*2*) or recombinant HC and LC. The mixtures were dialysed against 50 mM Tris-HCl (pH 8.4), 1 M NaCl, 2 M urea, and 20 mM DTT with stirring at 4°C for 18 h, followed by dialysis against 50 mM Tris-HCl (pH 8.4) and 600 mM glycine without agitation at 4°C for another 72 h, as d tailed previously (*33*).

The extent of formation of dichain species was monitored by SDS-PAGE in the absence of reducing reagent. The relative neuroparalytic activities of reconstituted preparations of recombinant and native dichain were assessed by measurement of neuromuscular paralysis *in vitro*, and determination of mouse lethality. For the former, mouse left phrenic hemi-diaphragm was dissected and maintained in the bath containing 10 ml KR buffer. After a stabilisation period of 10 min in the bath, each reconstituted sample or KR buffer (as a control) was added and nerve-evoked muscle contraction recorded as above. For quantitation of mouse lethality, toxin samples (maximum volume: 200 μl/mouse) were injected subcutaneously into the dorsal neck region of mice (4-5 weeks old with 20 g body weight, four mice/group) and the LD_{50} values (from three different experiments) determined, as described previously (*41*).

RESULTS

Characterisation of Plasmids Containing HC and H_C Coding Sequences of TeTx—The PCR amplified and purified H_N and H_C fragments of TeTx were digested with the appropriate restriction enzymes and subcloned into pMAL-c2-T and pMAL-c2, respectively. pMAL-HC was produced by ligating H_N and H_C into pMal-c2-T, as detailed in "MATERIALS AND METHODS." DNA encoding HC or H_C from positive colonies grown on LA plates containing ampicillin was purified using a DNA miniprep kit. DNA sequencing demonstrated that the two DNA fragments subcloned into pMAL vectors were in the correct reading frames with those for MCS, factor Xa and/or thrombin cleavage sites (Fig. 2, A and B). This creation herein of the modified pMAL-c2-T makes it possible to purify the HC fusion protein either on amylose resin *via* MBP (used in this study) or by chromatography on a nickel-containing column (*via* His-tag; data not shown); also, cleavage of MBP from the HC can be achieved with either factor Xa or thrombin. A major advantage of using thrombin is that efficient digestion can be accomplished at 4°C (see below) which minimises denaturation and maintains the solubility of HC which tends to become insoluble when incubated at higher temperatures for extended periods. In the case of MBP-H_C, the unmodified pMAL-c2 construct (Fig. 2B) was used because digestion with factor Xa at 22°C proved satisfactory due to the greater solubility of H_C.

Expression and Purification of r-HC and r-H_C—The resultant plasmids (pMAL-HC and pMAL-H_C) were transformed into *E. coli* JM109 and TGI, respectively, and induction of the fusion proteins was initiated by addition of IPTG. Affinity chromatography of the expressed protein was carried out as described previously (*33*) except that amylose resin was prepared rather than purchased; its binding capacity for the MBP-fusion proteins was similar to that of commercial resin. The expression and purification of MBP-HC and MBP-H_C were monitored by SDS-PAGE; Coomassie staining showed that MBP-HC or MBP-H_C appeared only in the lysates of cells induced by IPTG (Fig. 3, A and B). The bulk of each fusion protein became bound to the resin (not shown) and could be eluted from the amylose column with maltose (Fig. 3, A and B). Most of the MBP-HC was cleaved with thrombin after digestion at 4°C (Fig. 3A); at this low temperature the released HC remained soluble. Cleavage of MBP-H_C required incubation with factor Xa at 22°C (Fig. 3B). Free HC and H_C were separated from MBP by reabsorption of the latter onto new amylose columns and their identities were confirmed by Western blotting, using mouse anti-serum raised against native HC (Fig. 3, A and B). Microsequencing revealed that eight residues at the N-terminus of r-HC are identical to those of native HC, preceded by the four amino acids encoded by the MCS nucleotide sequence of pMAL-c2-T (Fig. 2C); nine

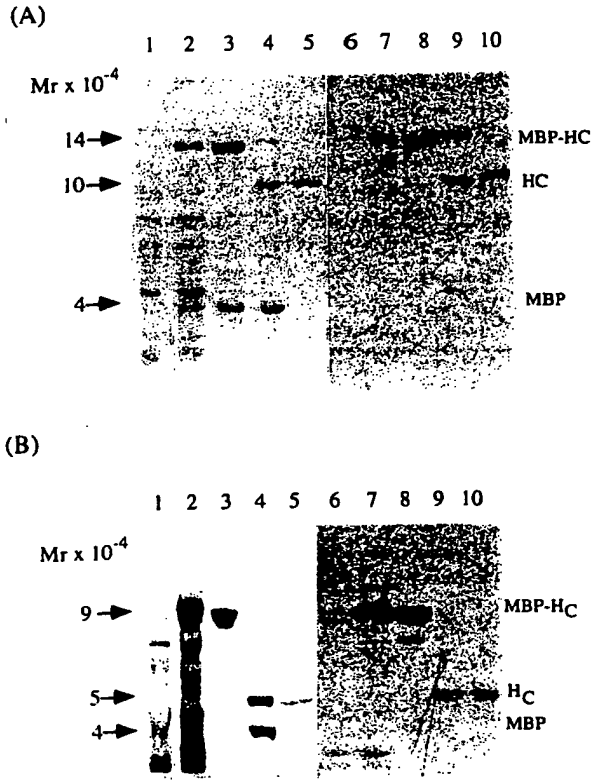

Fig. 3. **Expression and purification of r-HC and r-H_C monitored by SDS-PAGE and Western blotting.** *E. coli* was transformed with pMAL-HC or pMAL-H_C; after induction with IPTG, the bacteria were pelleted, resuspended in column buffer and lysed by sonication. The cell lysate was loaded onto a column containing the amylose affinity resin (40 ml). After washing away unbound contaminants with 320 ml of column buffer, the bound MBP-HC or MBP-H_C protein was eluted with 100 ml of column buffer (3 ml fractions) containing 10 mM maltose. The eluted fusion proteins (peak fractions) were concentrated in a Centricon (M_r 50K cut-off). MBP-HC and MBP-H_C were cleaved with thrombin at 4 C for 16 h, and factor Xa at 22°C for 24 h, respectively. After digestion, PMSF was added to a final concentration of 1 mM to inactivate the excess enzyme; MBP was removed by adsorption to a new lot of amylose resin. The samples were subjected to SDS-PAGE (8% polyacrylamide gel) with Coomassie staining (lanes 1-5) or detection with polyclonal anti-HC antibodies (lanes 6-10), by the procedure detailed elsewhere (*33*). Panels (A) and (B) show HC and H_C preparation, respectively. Lanes 1 and 6: lysate of cells not induced; 2 and 7: lysate of cells induced with IPTG; 3 and 8: fusion protein eluted from the amylose column; 4 and 9: fusion protein after incubation with thrombin or factor Xa to release toxin sample and MBP; 5 and 10: purified toxin sample. The sizes of MBP-HC, MBP-H_C, r-HC, r-H_C, and MBP, derived from the mobilities of standard proteins, are shown. Note: A low molecular weight band shown in the uninduced and induced samples in (B) was probably caused by non-specific binding of anti-HC antibody to an unknown bacterial protein and after bio-specific elution from the amylose resin, the stained band disappeared.

residues obtained from the N-terminus of r-H_C correspond to those in native H_C, preceded by six amino acids encoded by the MCS nucleotide sequence of pMAL-c2 (Fig. 2D). After purification, ~15 mg MBP-HC and ~23 mg MBP-H_C proteins were generally obtained from 1 liter of cell culture, respectively.

CD Spectra of Native HC and r-HC—The far UV CD spectrum of r-HC closely resembles that of its native counterpart (Fig. 4). The estimated secondary structure content for r-HC is 25% α-helix, 37% β-sheet, 7% β-turn, and 31% random coil. HC isolated from TeTx using 4 M urea has been reported (*44*) to give somewhat different values; the lower proportion of β-structure may be attributed to the complete and irreversible loss of toxicity of TeTx known to be caused by 4 M urea (*2*), unlike the milder conditions employed herein. Notably, a similar content of β-structure (40%) has been published (*4*) for the homologous HC of botulinum neurotoxin type A. Our results for the r-HC are similar to those reported by de Filippis *et al.* (*45*) for the LC (27% α-helix and 43% β-structure) of TeTx and suggest that both chains may hav similar secondary structures.

Antagonism of the Neuromuscular Blocking Action of TeTx by Native HC, r-HC, or r-H_C—It has been shown that pre-incubation of diaphragm with native HC or H_C derived from TeTx antagonises the neuromuscular blocking activity of TeTx by competing for acceptor sites on the nerve membrane (*13, 14*). This provided a means of assaying the biological activity of these recombinant proteins. Mouse phrenic nerve-muscle preparations were pre-equilibrated at 4°C for 60 min in mKR buffer alone (as a control) or with the sample specified, then incubated in the same buffer at

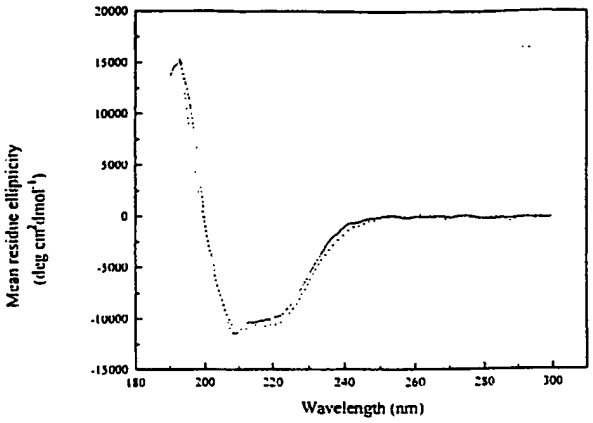

Fig. 4. **Far UV CD spectra of r-HC and native HC.** These spectra of HC were recorded on an Aviv 62DS instrument, with an acceptance angle ≥90°, fitted with a thermostatted cell holder, using a 1 mm pathlength suprasil cuvette. Data points were collected in steps of 0.2 nm, from 300 to 190 nm. UV CD spectra were recorded at 22 C for recombinant (----) and native ( ) HC at 0.10 mg/ml in 10 mM sodium phosphate buffer (pH 7.0).

TABLE I. **Relative abilities of HC and H_C to antagonise the blocking action of TeTx on neuromuscular transmission.**

Proteins added[a] (μM; final conc.)	Paralysis time[b] (min)	p value[c]
None	250±8	
Native HC (0.5)	369±13	<.01
r-HC (1.0)	380±9	<.01
r-H_C (2.0)	376±11	<.01
MBP (2.0)	246±10	

[a]Isolated mouse nerve-muscle diaphragm preparations were used. Control tissue was incubated without the fragments at 4°C whereas the experimental groups were pre-incubated at 4°C with test proteins in mKR buffer for 60 min, and then exposed at 4°C to 1 nM TeTx for an additional 30 min. All tissues were washed thrice with mKR and three times with KR medium. The temperature was raised to 37°C, the phrenic nerve stimulated supramaximally and paralysis time recorded as detailed in Ref. *42*. [b]Mean values (±SD) obtained from three different experiments. [c]Differences evaluated statistically using the Student's *t*-test.

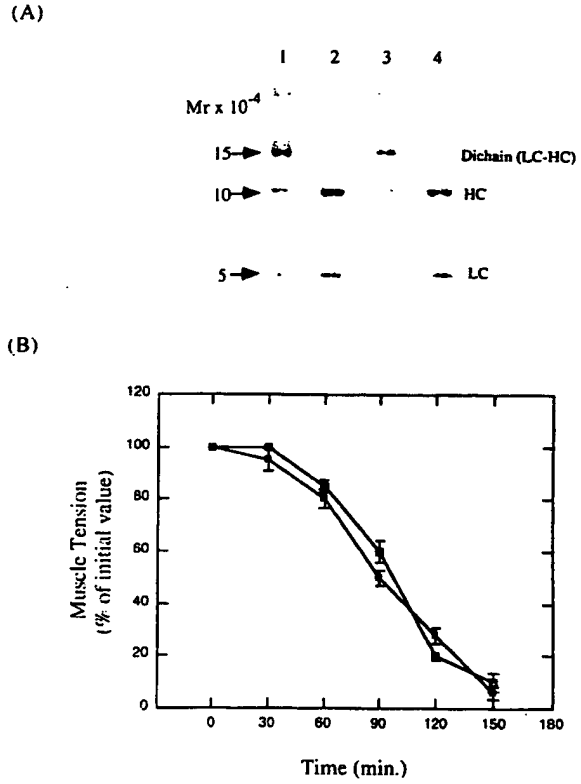

Fig. 5. **Formation of disulphide-linked dichain toxin following reconstitution of either recombinant LC and HC or their native counterparts: effects on mouse neuromuscular transmission.** Equimolar amounts of either recombinant LC and HC or their native equivalents were mixed together and dialysed against 50 mM Tris-HCl (pH 8.4), 1 M NaCl, 2 M urea, and 20 mM DTT with stirring at 4 C for 16 h, followed by dialysis against 50 mM Tris-HCl (pH 8.4) and 600 mM glycine without agitation at 4°C for 72 h, as detailed previously (*33*). (A) The formation of dichain was monitored by SDS-PAGE under non-reducing conditions. Lanes 1 and 2: dichain reconstituted from native LC and HC run under non-reducing (lane 1) and reducing (lane 2) conditions; lanes 3 and 4: dichain assembled from recombinant LC and HC run under non-reducing (lane 3) and reducing (lane 4) conditions. M_r values for dichain, HC and LC, determined from their mobilities relative to standard proteins, are indicated. (B) The inhibition of neuromuscular transmission by 20 nM native dichain (■) and 80 nM recombinant dichain (□) was assessed using the mouse hemi-diaphragm preparation. The left phrenic nerve was stimulated at 37°C and the muscle contraction and paralysis times recorded as before (*42*). Values are the means (±SD) obtained from three different experiments; the 100% value represents the initial muscle contraction evoked by supramaximal stimulation.

4°C with 1 nM TeTx for an additional 30 min. Compared to the control, the times taken for TeTx-induced paralysis were extended by each of the chain preparations but not by MBP (Table I); this demonstrated that the HC and H_C produced in *E. coli* possess the ability to bind to the ecto-acceptors. However, native HC was about 2-fold more potent than its recombinant counterpart in inhibiting nerve-evoked muscle tension; also, the potency of r-H_C was half that of r-HC (Table I). Not surprisingly, the native HC is the most efficient antagonist because of it being produced by *Clostridium tetani* as a single-chain protein that includes LC; this may help to induce a conformation of HC that is optimal for binding to the neuronal ecto-acceptors. In contrast, r-HC and r-H_C were produced in *E. coli* as a fusion products and had to be cleaved with proteases before being subjected to purification. To explore whether these various binding fragments could displace the bound toxin, a series of related experiments was carried out. After the diaphragms were first incubated in mKR buffer with 1 nM TeTx at 4°C for 30 min, to allow TeTx to become bound to the neuronal membrane ecto-acceptors, HC, r-HC, or r-H_C, were added and further incubated in the same buffer for 60 min at 4°C. The tissue samples were then washed with mKR and KR before the temperature was raised to 37°C and paralysis monitored, as before. The control diaphragms, incubated with 1 nM TeTx only but otherwise processed similarly, became paralysed in 240±10 min; however, the paralysis times for the experimental groups were not significantly different, demonstrating that neither H_C nor HC could displace the bound toxin.

Reconstitution of Recombinant Dichain and Assessment of Its Biological Activity In Vivo and In Vitro—To ascertain whether HC and LC made in *E. coli* can associate and form an interchain disulphide bridge, and to establish if the resultant dichain molecule retains its biological activity, equimolar amounts were mixed and dialysed against renaturing buffer. SDS-PAGE under non-reducing conditions revealed that reconstitution of the recombinant chains occurred but to a somewhat lesser extent than that observed for their counterparts isolated from TeTx (Fig. 5A; Table II). The biological activity of recombinant dichain was assessed *in vitro* on a mouse nerve-muscle preparation; 80 nM of dichain generated by the two recombinant chains caused neuromuscular paralysis within 150±10 min (Fig. 5B), representing 25% of the neuroparalytic activity exhibited by dichain formed with the individual chains isolated from natural TeTx. This is a very satisfactory lev l of activity for totally recombinant toxin particularly considering that the dichain made using recombinant LC and natural HC possesses lower activity than that reconstituted using both of the chains isolated from TeTx (*33, 34*). When the recombinant dichain was tested *in vivo*, it displayed appreciable lethality in mice (Table II), its toxicity being 300 ng/kg; thus, it is 40-fold less toxic in the whole animal than the dichain made from the natural chains, compared with only the 4-fold difference found using the neuromuscular paralysis assay. Most importantly, when sub-lethal doses of the recombinant dichain were injected in the neck region of mice, a spastic paralysis was induced; the symptoms produced were indistinguishable from those caused by native TeTx that results from blockade of transmitter release at inhibitory synapses in the spinal cord. However, when r-HC (10 μg) and r-LC (10 μg) were mixed together and immediately injected into mice, the recombinant chain mixture failed to produce typical tetanus symptoms *in vivo* (Table II), in keeping with the requirement for an intact disulphide bond between the LC and the HC. Thus, th dichain produced using both HC and LC synthesised in *E. coli* can be refolded in a conformation that is capable f ecto-acceptor binding, internalisation, axonal retrograde transport and blockade of the release of inhibitory transmitters in spinal cord inter-neurons.

TABLE II. **Mouse lethality of TeTx and dichains reconstituted from either native or recombinant chains.**

Samples[a]	Mouse lethality[b] (LD_{50}; ng/kg body wt)	Covalently linked dichain (%)
TeTx	1	–
Dichains reconstituted using:		
Native chains (HC & LC)	7.5	62[c]
Recombinant chains (HC & LC)	300	44[c]
r-HC and r-LC mixed but not reconstituted[d]	$>10^6$	–

[a]Equimolar amount of native or recombinant HC and LC were mixed and dialysed against 50 mM Tris-HCl (pH 8.4), 1 M NaCl, 2 M urea, and 20 mM DTT with stirring at 4°C for 16 h, followed by dialysis against 50 mM Tris-HCl (pH 8.4) and 600 mM glycine without agitation at 4°C for 72 h. [b]Mouse toxicity was defined as that amount of toxin (ng/kg) which kills 50% of injected mice within four days, as detailed previously (*41*). [c]The formation of dichain was monitored by SDS-PAGE in the absence of reducing reagent (Fig. 5) and the content of dichain species quantified by densitometric scanning of Coomassie stained gels. [d]Recombinant chains [HC (10 μg) and LC (10 μg)] which were mixed together just before being injected into mice. Mean values are shown from at least three different experiments.

DISCUSSION

The successful high level expression of r-H_C and r-HC, for the first time, allowed examination of their effects on TeTx-induced neuromuscular paralysis; also, the recombinant technology established herein offers the important advantage of avoiding the difficulty of removing trace amounts of contaminating LC or dichain from HC or H_C, as normally isolated from the native toxin. The r-HC and r-H_C preparations were non-toxic but, like their native counterparts (*13, 14*), antagonised the neuroparalytic activity of TeTx by competing for binding to ecto-acceptors and, thus, delaying the subsequent internalisation and intracellular action of TeTx. Neither native HC, r-HC, nor r-H_C was capable of slowing neuromuscular paralysis after TeTx had first bound to the target cells; this is indicative of very tight binding of the native toxin, though the precise nature of the ecto-acceptor still remains unclear. It has been proposed that gangliosides can act as receptors for TeTx (*15, 46*) but proteins are also thought to be involved in its neuronal binding (*47, 48*). Such formation of toxin complexes with ganglioside and protein receptor(s) has been suggested to underlie the specific high-affinity binding that culminates in internalisation and translocation (*49*). The twofold higher potency of native HC relative to r-HC accords with the fact that native HC is produced and folded, under optimal conditions, as a single chain; later, it is cleaved to yield the dichain toxin. The lower binding efficiency of r-HC could arise from less perfect folding because of being produced in *E. coli* as a fusion and exposed to enzymatic cleavage and a lengthy purification process; however, there was no significant difference between the CD spectra of r-HC and native

HC indicating that their gross folded structures are similar, consistent with the acceptable level of biological activity observed for the recombinant material. Notably, r-H_C proved less effective than r-HC in retarding the neuromuscular paralysis caused by TeTx, possibly because the latter adopts a more native conformation due to the presence of the H_N fragment and/or the H_N may contribute to the ecto-acceptor binding. Although the native HC, r-HC, and r-H_C delayed the onset of paralysis by TeTx of neuromuscular junction, the antagonism exhibited was not complete even when a vast molar excess of each over TeTx was used. This might be explained by the fact that these suboptimally folded fragments could not saturate all the TeTx acceptors on the nerve terminals; therefore, the highly potent TeTx may still interact with the acceptors *via* a very limited number of unoccupied sites and subsequently block transmitter release.

It was also demonstrated, for the first time, that r-HC can be successfully reconstituted with expressed LC, forming a disulphide-bridged dichain. The resultant complete recombinant toxin proved to be only 4-fold less potent than the two reconstituted native chains. However, when tested *in vivo*, the recombinant dichain proved 40-fold less toxic than its native equivalent; this may be indicative of a lower amount of recombinant protein being efficiently transported into CNS, the main target for TeTx. Less perfect folding of the recombinant chains themselves may account for the lower percentage of dichain formation, and this may also increase its susceptibility to degradation *in vivo*, resulting in the reduced lethality observed. In contrast, free r-LC and r-HC when briefly mixed together before injection did not exhibit any toxicity in mice, even when large quantities of each were employed. This clearly indicates that an intact interchain disulphide bridge between LC and HC is required, a bond which cannot form simply by mixing the two separate chains together for a short time. This emphasises the importance of the disulphide bridge in the membrane translocation of TeTx LC into the cytosol of targetted neurons, a result previously observed (*50*).

The novelty of this study is that functional dichain has been produced from r-HC and r-LC, expressed separately in *E. coli*. A CNS-directed drug-delivery vehicle based upon a recombinant dichain, formed from r-HC and an enzymatically-inactivated isoform of r-LC (*33*), is now feasible. Additionally, the expressed non-toxic whole protein may produce better immunological protection against tetanus than chemically-inactivated TeTx. Indeed, the innocuous dichain toxin gave a higher antibody titre than that of r-H_C alone (*28*); also, antibodies raised against the LC were found to give protection against challenge with TeTx (*28, 51*). Thus, non-toxic mutants of TeTx have the potential to provide improved vaccines for tetanus which kills millions of people yearly; these would be safer to handle, easier to produce and could be purified by affinity- rather than conventional-chromatography. Lastly, the proven feasibility of producing functional r-HC and r-H_C provides scope for investigating the HC and H_C by site-directed mutagenesis to elucidate their roles in binding, internalisation and retrograde transport of TeTx.

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